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ELISA for the Detection of Serum and Saliva IgA Against the BMRFI Gene Product of Epstein-Barr Virus

E.C.B. Nadala, T.M.C. Tan, H.M. Wong, and R.C.Y. Ting

Institute of Molecular and Cell Biology, National University of Singapore, Singapore (E.C.B.N., T.M.C.T., R.C.Y.T.); Department of Virology, Cancer Institute, Sun Yat-Sen University of Medical Science, Guangzhou, People's Republic of China (H.M.W.)

The BMRF1 protein is an Epstein-Barr virus (EBV) DNA polymerase accessory protein that forms part of the early antigen diffuse (EA-D) component. An enzyme-linked immunosorbent assay (ELISA) was developed for the detection of IgA antibody to the BMRF1 protein of EBV in saliva and serum samples. The assay was shown to be specific for nasopharyngeal carcinoma (NPC) patients and, when used with saliva alone, to have a sensitivity comparable to an existing indirect immunoperoxidase assay for early antigens. The sensitivity of the assay could be significantly enhanced to 86% by the use of paired saliva and serum samples.

KEY WORDS: cancer, diagnosis, herpesvirus, immunoassay

INTRODUCTION

Epstein-Barr virus (EBV) has long been suspected of playing a role in the etiology of nasopharyngeal carcinoma (NPC) [Henle et al., 1970]. NPC is rare in North American and European Caucasians. In contrast, it is relatively common in southern China, with a standardized annual incidence of 26 per 100,000 males and 10 per 100,000 females. All anaplastic or low-differentiated NPCs, irrespective of geographical or ethnic origin, carry the EBV DNA [Klein et al., 1974; Andersson-Anvert et al., 1977]. EBV infection is latent and the viral DNA is a clonal episome. The clonality of EBV DNA in NPC arises from the clonal expansion of a single EBV-infected cell [Raab-Traub and Flynn, 1986].

Serum IgA antibodies to various EBV antigens, notably the virus capsid antigen (VCA) and early antigens (EA), are known to be elevated in NPC and are considered to be useful diagnostic markers for the disease [Henle and Henle, 1976; Wara et al., 1975]. Over the years, various recombinant protein-based ELISA tests for anti-EBV antibodies have been reported [Foong et al., 1990; Joab et al., 1991; Pothen et al., 1993; Mathew et al., 1994]. Although many of these tests have good sensitivity, most are not specific for NPC. Anti-EA IgA is known to be a highly specific marker for NPC. Common methods to detect it at present (immunofluorescence or immunoperoxidase assays), however, still rely on the use of lymphocytes that make the tests more costly and complicated to perform. Hence, the development of a simpler and cheaper ELISA will be highly desirable and particularly useful for population-based screening to detect preclinical NPC.

The BMRF1 open reading frame of the EBV codes for a protein that forms a part of the early antigen diffuse (EA-D) component [Li et al., 1987]. This protein has been expressed in insect cells using the baculovirus system, purified, and characterized as an EBV DNA polymerase accessory protein [Tsurumi, 1993].

We describe a promising new ELISA for anti-BMRF1 IgA antibody that may be particularly useful in the diagnosis of NPC using saliva samples.

MATERIALS AND METHODS

Sera and Saliva

Sixty-six samples of NPC sera and 143 samples of NPC saliva were used in this study. Five of the sera and three of the saliva samples were from NPC patients in Singapore. The rest of the NPC sera and saliva samples were from patients in Guangzhou, PRC. Thirty-five serum samples from normal healthy people, 52 serum samples from cervical carcinoma patients, and 72 saliva samples from normal healthy people were also collected as control. Saliva samples were collected using the Omni-Sal Saliva Collection Device (Saliva Diagnostic Systems Pte. Ltd., Singapore).

BMRF1 Protein

The BMRF1 open reading frame was amplified by polymerase chain reaction (PCR) from B95-8 DNA,

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Address reprint requests to Theresa Tan, Institute of Molecular and Cell Biology, National University of Singapore, 10 Kent Ridge Crescent, S0511, Singapore.

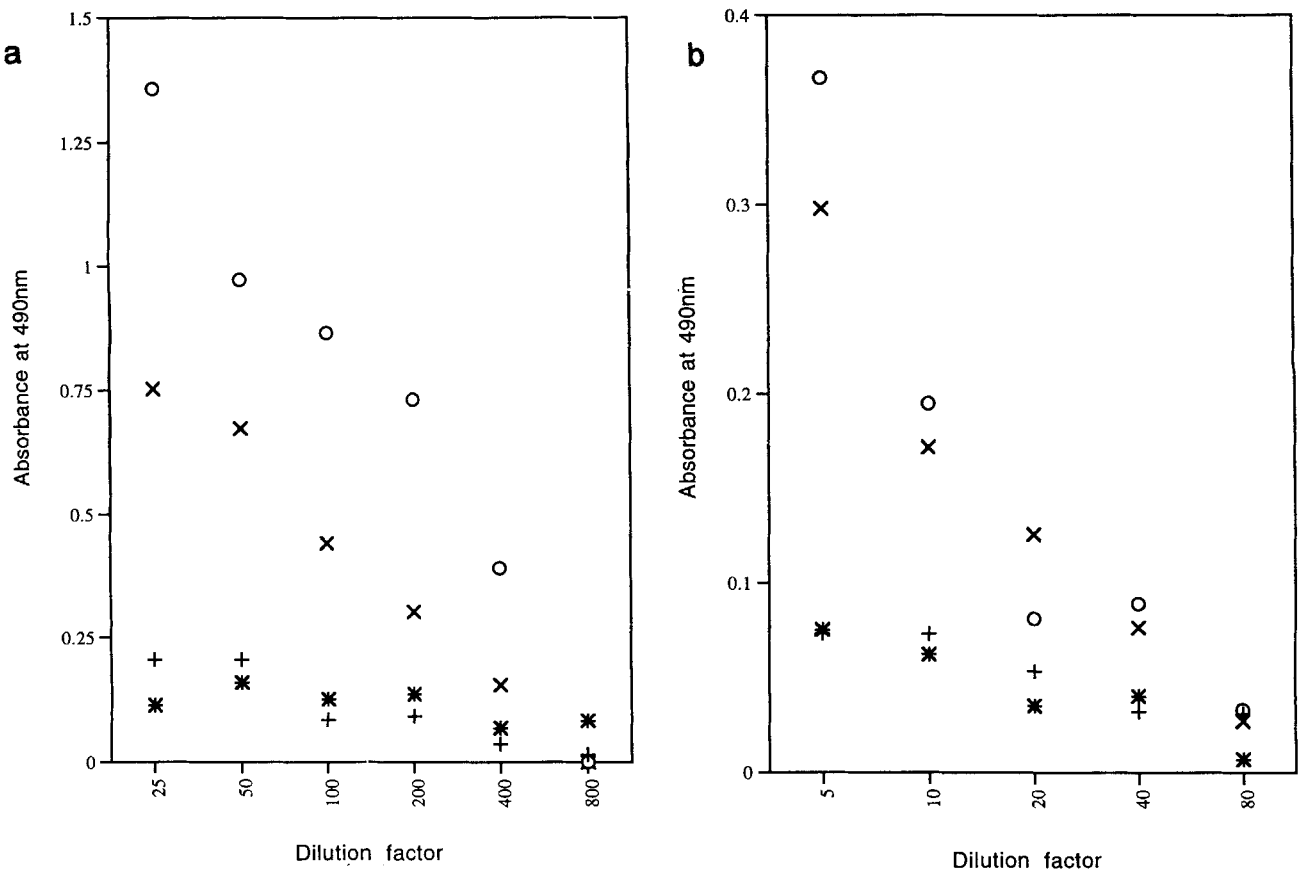

Fig. 1. **a:** Serum anti-BMRF1 IgA antibodies in normal healthy people (+, *) and NPC patients (○, ×).
b: Saliva anti-BMRF1 IgA antibodies in normal healthy people (+, *) and NPC patients (○, ×).

cloned into the pMalc2 expression vector (New England Biolabs, Beverly, MA) as an MBP fusion protein and expressed in the BL-21 protease-deficient *Escherichia coli* strain. The protein was purified as a soluble 86 kd MBP-BMRF1 fusion protein by affinity purification using amylose columns.

ELISA

The recombinant MBP-BMRF1 and MBP (New England Biolabs) proteins were diluted to 10 μg/ml in 50 mM $NaHCO_3$, pH 9.6, and 50 μl was applied to each microtiter plate well (Nunc-Immuno Plates, Nunc, Denmark). Plates were incubated overnight at 4°C, washed twice with phosphate-buffered saline (PBS; pH 7.3), dried for 1–2 hr at 37°C and stored at 4°C. Wells were blocked with 300 μl of 5% skim milk in PBS (S-PBS) for 1 hr at room temperature (RT). Sera and saliva samples were tested in triplicate. After five washings with 0.05% Tween-20 in PBS (PBS-T), a peroxidase-conjugated rabbit anti-human IgA (Dako, Glostrup, Denmark) diluted 1:4,000 in 1% S-PBS was added at 50 μl per well. Plates were incubated for 2 hr at RT, then washed five times with PBS-T. The enzyme substrate OPD (1,2-phenylenediamine; Dako) was added at 100 μl per well, incubated for 30 min at RT, and stopped with 100 μl per well of 1 N H_2SO_4. Absorbance was read using the Dynatech MR7000 ELISA machine (Dynatech, Hong Kong) at 490 nm with the reference filter set at 630 nm. All plates included triplicate wells containing an identical positive NPC serum sample and PBS as positive and negative controls, respectively. A serum sample was considered positive if the difference of the average readings for that sample in MBP-BMRF1 and MBP wells was 0.14 or above. A saliva sample was considered positive if the difference was 0.1 or above.

Indirect Immunoperoxidase Assay (IPA)

The IPA test for anti-EA IgA was carried out as previously described [Li et al., 1991]. Briefly, acetone-fixed Raji cells were incubated for 45 min at 37°C with dilutions of human serum samples, washed, incubated for 45 min at 37°C with anti-human IgA conjugated to peroxidase, washed, and then stained with diaminobenzidine (DAB).

RESULTS

A series of initial experiments were carried out to determine the most suitable dilution for serum and saliva samples to be used for the anti-BMRF1 IgA assay. Serum and saliva samples from two patients and two

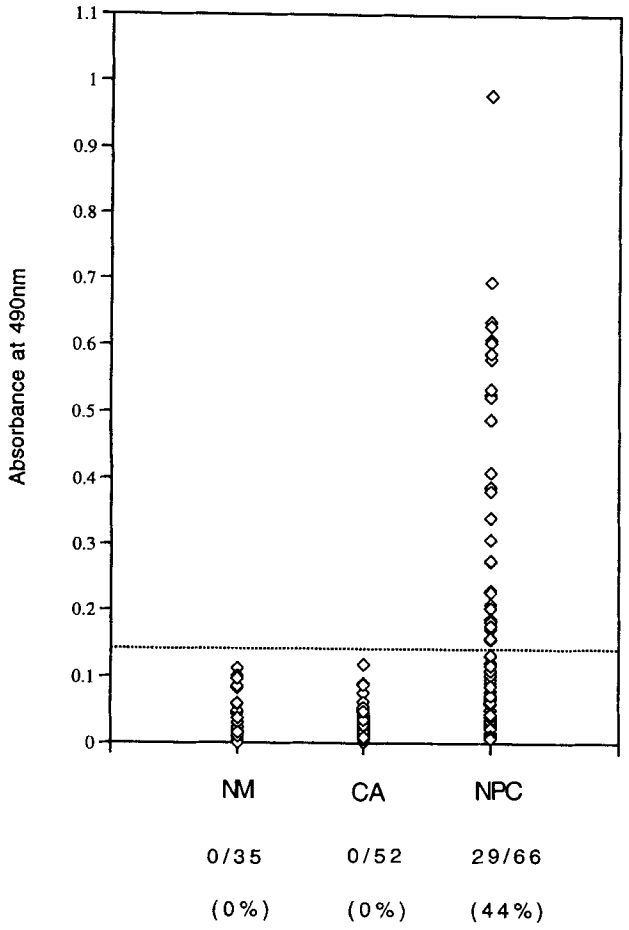

Fig. 2. Distribution of serum IgA antibodies to BMRF1 protein of EBV in normal healthy people (NM), cervical carcinoma patients (CA), and NPC patients. Values below the graph show the relative numbers of positive samples.

TABLE I. Results of Anti-BMRF1 ELISA IgA Assay Using Saliva

Category	Number of saliva assayed	Number of positives (percent)
Healthy	72	4 (6%)
NPC	143	72 (50%)

healthy controls were assayed. The results, shown in Figure 1, indicate that a 1:100 dilution was most suitable for serum samples, while a 1:10 dilution was suitable for saliva.

One hundred fifty-three serum samples were tested for anti-BMRF1 IgA. The results are shown in Figure 2. None of the 35 sera from normal healthy people and 52 sera from cervical carcinoma patients were positive. Anti-BMRF1 IgA was positive in 29/66 (44%) NPC sera.

Two hundred fifteen saliva samples were tested for anti-BMRF1 IgA. The results are shown in Table I. Only four of the 72 saliva samples from normal healthy people were positive, whereas 72/143 (50%) NPC saliva samples were positive.

Of the NPC sera and saliva samples, 42 were paired samples from patients who had been diagnosed histologically. The results of the assays for this particular group of patients are shown in Table II. When analyzed as a group, 43% (18/42) of the serum and 69% (29/42) of the saliva samples were positive for anti-BMRF1 IgA. However, 62% (26/42) of the serum samples were positive for anti-IgA by IPA. When the ELISA results were combined, 79% (33/42) of the NPC patients were positive for either one or both of the serum and saliva tests. In addition, when these results were combined with those of the IPA, up to 86% (36/42) of the patients were shown to be positive by one of the three tests.

DISCUSSION

The results of this study show clearly that the ELISA for anti-BMRF1 IgA was very specific. None of the control sera was positive, while only 6% of control saliva was positive with this test.

The sensitivities of the ELISA using serum and saliva were 44% and 50%, respectively. These figures, however, were derived from testing of sera and saliva of diagnosed NPC patients who had not all been confirmed by histological examination. In addition, some of the sera and saliva samples were not paired samples.

In order to make more meaningful comparisons between the ELISA and the IPA, 42 paired samples from patients who had been diagnosed histologically were analyzed. One unusual result of this analysis was the significantly higher positive result seen for the saliva test in this subgroup (69% vs. 50%). We have no explanation for this discrepancy at the moment other than to speculate that some of the NPC cases had been misdiagnosed.

Although ELISA was less sensitive than IPA when testing serum samples (43% vs. 62%), it compares quite favorably when testing saliva (69% vs. 62%). The lower sensitivity of this assay as compared to IPA when using serum samples may be due to either the dilution difference or the fact that the IPA test includes other components of the EA complex besides BMRF1.

From Table II, it is apparent that the results of the saliva test do not necessarily correlate with the results of the serum tests. Some patients were positive only in their saliva and not their sera or vice versa. The reason for this is not clear at present, but it certainly illustrates the importance of using saliva samples for NPC diagnosis either on their own or, when possible, as a supplement to the existing IPA tests. Whereas only 69% of the NPC saliva samples were positive when tested alone, this figure could be brought up to 79% when supplemented with results from serum testing by ELISA and up to 86% with the addition of results from IPA tests.

The results presented here demonstrate the potential for an ELISA using recombinant BMRF1 proteins in the testing of NPC saliva. In its present form, the ELISA may have the potential for use in a screening study of high-risk populations for preclinical NPC.

We are currently developing modified versions of this assay that could enhance the test sensitivity without

TABLE II. Comparison of the Anti-EA (IPA) or Anti-BMRF1 (ELISA) IgA Assays Using Paired Sera and Saliva of NPC Patients With Histological Diagnosis of Poorly Differentiated Nasopharyngeal Carcinoma or Poorly Differentiated Squamous Cell Carcinoma of the Nasopharynx

Assays	Number of patients positive for the assays
IPA and ELISA (sera) and ELISA (saliva)	11
IPA and ELISA (serum) only	3
IPA and ELISA (saliva) only	9
ELISA (serum) and ELISA (saliva) only	3
ELISA (serum) only	1
ELISA (saliva) only	6
IPA only	3
Not positive for any assay	6
Total	42

sacrificing specificity by the use of other recombinant EBV proteins.

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